PHYSICO-CHEMICAL STUDIES ON THE METHACYCLINE-TRIS[HYDROXYMETHYL]-AMINOMETHANE SYSTEM

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The MOTC-TRIS system was studied potentiometrically, cryoscopically and photometrically.

Potentiometric titration of MOTC with TRIS revealed one larger and seven smaller potential changes in the mV *vs.* 10^C curves. These changes occurred at identical TRIS:MOTC molar ratios, whatever the MOTC or TRIS concentration was. Accordingly, it may be supposed that one MOTC molecule may bind a maximum of five TRIS molecules.

As to the nature of the bond between MOTC and TRIS, the experimental and calculated cryoscopic data suggest it to be ionic.

Spectroscopic investigation gave some evidence that the binding of MOTC to protein was not hindered by the presence of TRIS. According to the increase in $d\varepsilon_{max}$, TRIS may enhance the ionization of MOTC.

As well known, tetracyclines are a group of broad-spectrum antibiotics, widely employed in medical practice. The tetracyclines are amphoteric compounds and therefore tend to be soluble in aqueous acid or aqueous base, but they exhibit extremely low solubility near their isoelectric points. The requirement for general medical employment, especially for injections, is a sufficiently high concentration and neutral pH. One way of meeting this requirement is to form derivatives with higher solubility in near-neutral or slightly-basic solution. For this purpose either the complex-forming ability [1, 2] or the chemical reactions of the amide group of the tetracyclines [3] was utilized. These derivatives showed increased solubility at pH 4.0—8.0, enhanced stability against alkalis, and reduced acute toxicity, tissue irritation and rapid tissue diffusion.

The methacycline (MOTC) prepared by Blackwood *et al.* [4] has come into increasing therapeutical use because of its high antibiotic activity. Kahán and Hammer [5, 6] have given an account of observations that the concentration of tetracyclines in aqueous solution of tris[hydroxymethyl]-aminomethane (TRIS) could be as high as 10 per cent at ph 7.4—7.8; this mixed solution exhibited high antibiotic activity when examined *in vitro* or *in vivo* [7].

In the present paper this MOTC—TRIS system was studied to obtain information about the interaction between MOTC and TRIS. For this purpose potentiometric, cryoscopic and photometric experiments were carried out on the MOTC—TRIS system.

Materials and methods

The 6-methylene-5-hydroxytetracycline hydrochloride (methacycline hydrochloride, MOTC) was kindly supplied by the Pfizer Corporation, Eastern Europe Division, Belgium. The tris[hydroxymethyl]-aminomethane (TRIS), A grade, Lot No. 100909 and bovine plasma albumin, A grade, Lot No. 64761 were obtained from Calbiochem. These chemicals were used without further purification.

The potentiometric experiments were carried out with a RADIOMETER pH-Meter 26, together with a TITRATOR 11, an ABU 12 autoburette and an SBR 2 titrigraph.

The freezing point depressions were measured with a KNAUER Electronic Temperature Measuring Instrument, using a KNAUER Thermoelectric Cooling Unit equipped with a precision thermistor as temperature sensor. Throughout these experiments care was taken to use the same measuring vessel and identical sample volume. The spectra were determined with a BECKMAN DU Spectrophotometer, using 1 cm silica cells.

Results and discussion

Potentiometric experiments

To investigate the effect of TRIS on the MOTC molecules, two pH-titrations were carried out, using a glass electrode, with almost the same concentrations of MOTC, 0.1216 g/25 ml and 0.1291 g/25 ml, respectively. 0.5 M TRIS was used as titrant in both cases, and the recorder sensitivity was adjusted to 0.5 and 0.2 pH units/cm, respectively. The change in potential with volume of titrant added is small, except in the region of titration where some binding between the two molecules can be expected and a relatively great change in potential takes place. This means that the number of greater changes in the potential corresponds to the number of TRIS molecules bound to the MOTC molecule. The titration curves obtained are very similar to the common acid-base titration curves (Fig. 1), with one equivalence point. Accordingly, the molar rations of MOTC and TRIS were 0.99 and 0.98, respectively. No other pH jump was observed on the titration curves, and therefore a series of potentiometric titrations were run, using a plain platinum electrode against a calomel electrode as reference. In these experiments MOTC solutions

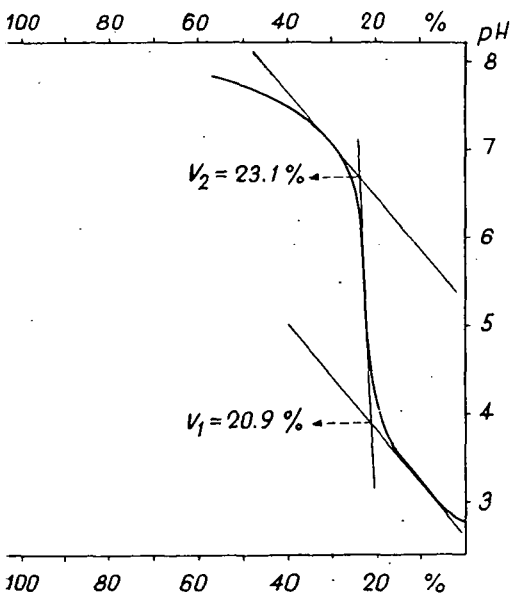

Fig. 1. pH-metric titration of MOTC; capacity of autoburette 2.5 ml (1 % = 0.025 ml)

of increasing concentration were titrated with 0.05, 0.1, and 0.5 M solutions of TRIS. Since the curves obtained were of similar type, it is sufficient to show only one (Fig. 2). Each curve consists of steep and mildly curving sections. This means that only one greater change in the potential took place due to the reaction of one molecule of TRIS with one molecule of MOTC, in agreement with the pH-titration results. Analysis of the region of small potential change was attempted *via* the first differential of the titration curve, but we failed to locate any other equivalence point owing to the extremely small potential change. Therefore, another method of analysis was used. The function between the potential and the logarithm of concentration being linear, a straightline plot is expected from exponential transformation. Thus, the potential data of the original curves were plotted against 10^C where C is the concentration, and the line of best fit was drawn using the least squares technique. All calculations were carried out with a type CII—10010 computer. As the same pattern was always obtained, only one plot is demonstrated (Fig. 3). Assuming that every change in the slope indicates further reaction of TRIS and calculating the corresponding concentrations, some information as to the number of bound TRIS molecules and the molar ratio of TRIS: MOTC may be expected. The results can be seen in Table 1. The greater change in the potential is caused when the quantities of TRIS and MOTC are equivalent, again in agreement with the results of pH-titration. These results show striking similarity to common acid-base titration, *i.e.* MOTC as an acid is titrated with TRIS as a base, and one molecule of MOTC forms a salt with one molecule of TRIS.

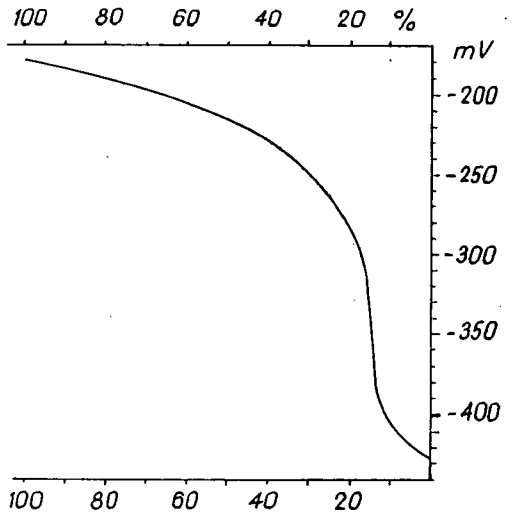

Fig. 2. Methacycline titrated with 0.1 M tris[hydroxymethyl]-aminomethane. Measured quantity 0.0172 g in distilled water Starting potential 430 mV; capcity of autoburette 2.5 ml (1 % = 0.025 ml)

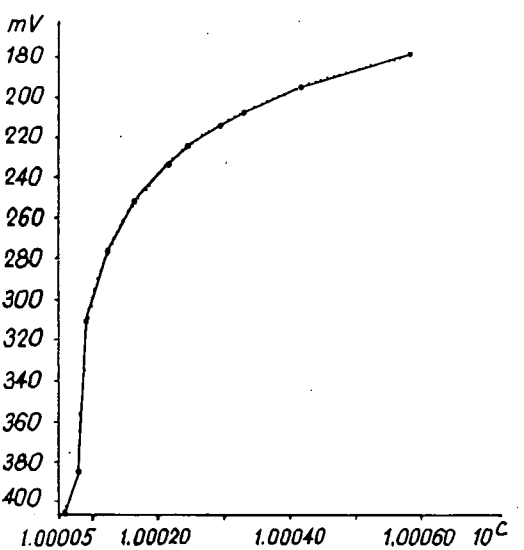

Fig. 3. 0.0172 g of MOTC (0.0000359 Mol) titrated with 0.1 M TRIS, plotted in mV *vs.* 10^C

The other changes in the potential, and accordingly in the slope, are much smaller but well discernible. The TRIS: MOTC molar ratios evaluated from the corresponding concentration of TRIS at these smaller changes of slope were 1.5, 2.0, 2.5, 30. 3.5, 4.0 and 5.0, respectively.

Cryoscopic experiments

From the potential *vs.* 10^C curves, the changes in the slopes were found to be increasingly smaller, which means that the bond between the MOTC and TRIS becomes weaker up to the fifth molecule of TRIS, whatever the nature of the bond itself. To throw light on this nature, a series of cryoscopic experiments were carried out. In these experiments first the freezing point depression of the pure solution of MOTC was measured at a given concentration. Then, increasing quantities of TRIS were added to the MOTC solution of the same concentration as before, so that the total TRIS concentration was twice, 2.5 times, 3.0 times, 3.5 times and 4.0 times that of the MOTC, keeping the volume and the concentration of MOTC constant. The freezing point depression was measured again. It should be noted that molality was used throughout these epxeriments. In addition the freezing point depressions were calculated theoretically, using the molecular freezing point depression value of 1.86 for water and the molal concentrations of all the molecular species separately. The values thus obtained were summed for each solution. The results can be seen in Table 2. Comparing the experimental and the calculated values in the case of a pure solution of MOTC, it was found that the former is twice the latter. This means that even in medium dilution the molecule undergoes complete dissociation to two ions, very probably $(MOTC)^+$ and Cl^-. In the further calculations, therefoer this dissociation was taken into consideration and the value for the Cl^- ion was added to the others. Comparison of the measured and calculated values now revealed good agreement for the MOTC—TRIS systems. This seems interesting because it means that in aqueous solution all of the bound TRIS molecules may dissociate and a highly ionized molecule of MOTC is formed. However, the possibility may arise that the TRIS molecules remain bound and their ionic dissociation contributes to the freezing point depression. This was settled in a separate experiment in which the freezing point depressions of pure TRIS solutions in various concentrations were determined. The experimentally found and calculated freezing point depression values are shown in Table 3, together with the increments in mole percent due to the ionic dissociation of the TRIS molecule. The increments were calculated by taking the given molality as one hundred percent and comparing this with the molality obtained from the measured depression of the freezing point. From the good agreement between the calculated and experimental freezing point depressions of the MOTC—TRIS system seen in Table 2, however, the conclusion may be drawn that the ionic dissociation of the TRIS molecule itself contributes little or not at all to the depression of the freezing point.

Spectroscopic experiments

Some spectroscopic experiments were carried out, partly to see the effect of TRIS on the light absorption of MOTC, and partly to obtain some information as to whether the TRIS exerts any influence on the bond between MOTC and protein. In these experiments care was taken that the pH's of the solutions to be

investigated should be practically identical. All of the solutions were therefore made in 0.02 M phosphate buffer, with a final pH 7.3—7.4. At such a phosphate concentration it was found that TRIS in a concentration of $2.5 \cdot 10^{-4}$ M did not have a substantial effect on the pH. This precaution was deemed necessary owing to the pH-dependence of the spectra of tetracyclines. Neither was any substantial change in pH found in the presence of MOTC at a concentration of $2.5 \cdot 10^{5}$ M. This MOTC concentration was used throughout all spectroscopic experiments. Thus the TRIS concentration, if present, was ten times that of the MOTC. Under these conditions, after determining the spectra of MOTC without and with TRIS, no change was found between 400 and 290 nm (wavelengths of maxima: 359 and 353 nm, with the optical density, $\varepsilon = 0.315$ at both wavelengths).

For the investigation of the bond between MOTC and protein, the best method seemed to be the evaluation of difference spectra [8]. The phosphate, MOTC and TRIS concentrations, and also the pH's were the same as before. The following spectra were determined separately: MOTC in phosphate buffer with and without TRIS, and the same with and without added albumin. The albumin concentration was $5 \cdot 10^{-5}$ M, *i.e.* twice that of the MOTC. Finally the absorption spectrum of albumin in the same concentration was also determined in phosphate buffer with and without TRIS. The given concentrations were kept constant in all solutions. The difference spectra themselves were obtained in the following way. First the values of the optical densities of the spectra of albumin with and without TRIS were subtracted from those of the spectra of MOTC with and without TRIS containing albumin at identical wavelengths. The differences thus obtained were again subtracted from the values of the optical densities of the spectra of MOTC in phosphate buffer with and without TRIS containing no albumin at all at the same wavelengths as before. These differences were plotted against wavelength (Fig. 4). The wavelengths of the maxima proved to be identical, but $d\varepsilon_{max}$ increased somewhat in the presence of TRIS. We may thus draw the conclusion that the presence of TRIS does not inhibit the binding of MOTC to protein. The higher $d\varepsilon_{max}$ may be attributed to the somewhat higher ionization of MOTC.

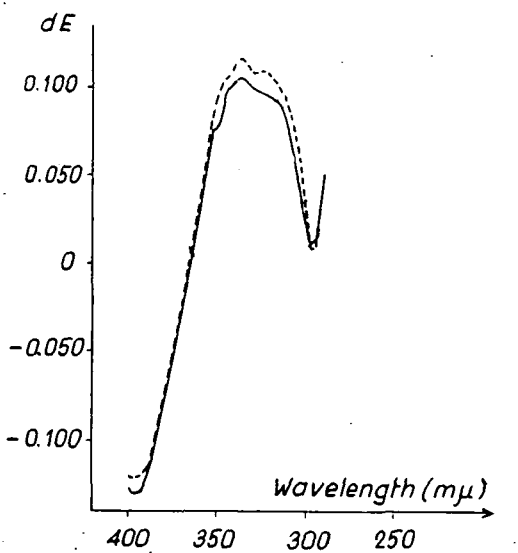

Fig. 4. Difference spectra of MOTC-albumine system: ——— without TRIS; ------- with TRIS

Table I

TRIS:MOTC molar ratios calculated from the mV vs. 10^c curves

TRIS molarity	0.05	0.05	0.1	0.1	0.1*	0.5	0.5	0.5
Grams of MOTC·10^{-3}	9.78	11.3	17.2	23.5	34.5	96.1	94.0	100.8
MOTC molarity·10^{-5}	2.04	2.36	3.59	4.9	7.2	20.0	19.63	21.05
Molar ratios	1.051	1.016	1.072	1.080	0.992	1.045	1.090	1.045
	1.418	1.546	1.489	1.487	1.485	1.432	1.548	1.520
	2.054	2.033	1.999	1.976	1.985	2.057	1.959	1.995
	2.495	2.500	2.589	2.567	2.471	2.507	2.427	2.446
	3.106	2.923	2.979	3.077	3.026	3.070	3.026	2.992
	3.473	3.432	3.508	3.545		3.470	3.530	3.553
	3.962	3.995	3.968	3.953		3.970	4.126	3.957
	4.867	5.000	4.998	4.809		4.970	5.114	4.973

* The measured quantity of MOTC turned out to be too large in relation to the concentration of TRIS, so the capacity (2.5 ml) of the autoburette ran out before the end of the titration.

Table II

Values of freezing point depression

$(MOTC)^+$ Molality	Cl^- Molality	TRIS Molality	Depression of freezing point in °C	
			Calculated	Found
0.0120	0.0120	—	0.0447	0.0435
0.0120	0.0120	0.0240	0.0894	0.0892
0.0120	0.0120	0.0301	0.1007	0.0988
0.0120	0.0120	0.0361	0.1119	0.1101
0.0120	0.0120	0.0422	0.1232	0.1216
0.0120	0.0120	0.0482	0.1344	0.1343

Table III

Values of freezing point depression

Depression of freezing point in °C		Concentration of TRIS in molality		Mole precent increment
Found	Calculated	Measured	Calculated	
0.1049	0.0950	0.0511	0.0564	10.4
0.0876	0.0782	0.0420	0.0471	12.0
0.0814	0.0670	0.0360	0.0438	21.5
0.0395	0.0223	0.0120	0.0212	76.8

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ФИЗИКО-ХИМИЧЕСКОЕ ИЗУЧЕНИЕ СИСТЕМЫ МЕТАЦИКЛИН—ТРИС(ГИДРОКСИМЕТИЛ) АМИНОМЕТАН

Г. Л. Сепеши, И. Л. Кахан

Проведено потенциометрическое, криоскопическое и фотометрическое изучение системы МОТЦ-ТРИС.

Потенциометрическое титрование МОТЦ-а ТРИС-ом дало один большой и семь менее значительных скачков потенциала на кривых мв. -10^{c}. Эти изменения наблюдались при одинаковых мольных соотношениях МОТЦ-а и ТРИС-а, независимо от их концентрации. На основании этих данных предположено, что одна молекула МОТЦ-а может связывать максимально пять молекул ТРИС-а.

Полученные экспериментальные и расчетные криоскопические данные позволяют предположить наличие ионной связи между молекулами МОТЦ-а ТРИС-а.

Спектроскопическими измерениями показано, что связывание МОТЦ-а к белку не затрудняется присутствием ТРИС-а. Увеличение значений $d\varepsilon_{\text{макс}}$ указывает на вероятность того, что в присутствии ТРИС-а степень ионизации МОТЦ-а возрастает.